Diversity in sporulation and spore properties of foodborne Bacillus strains

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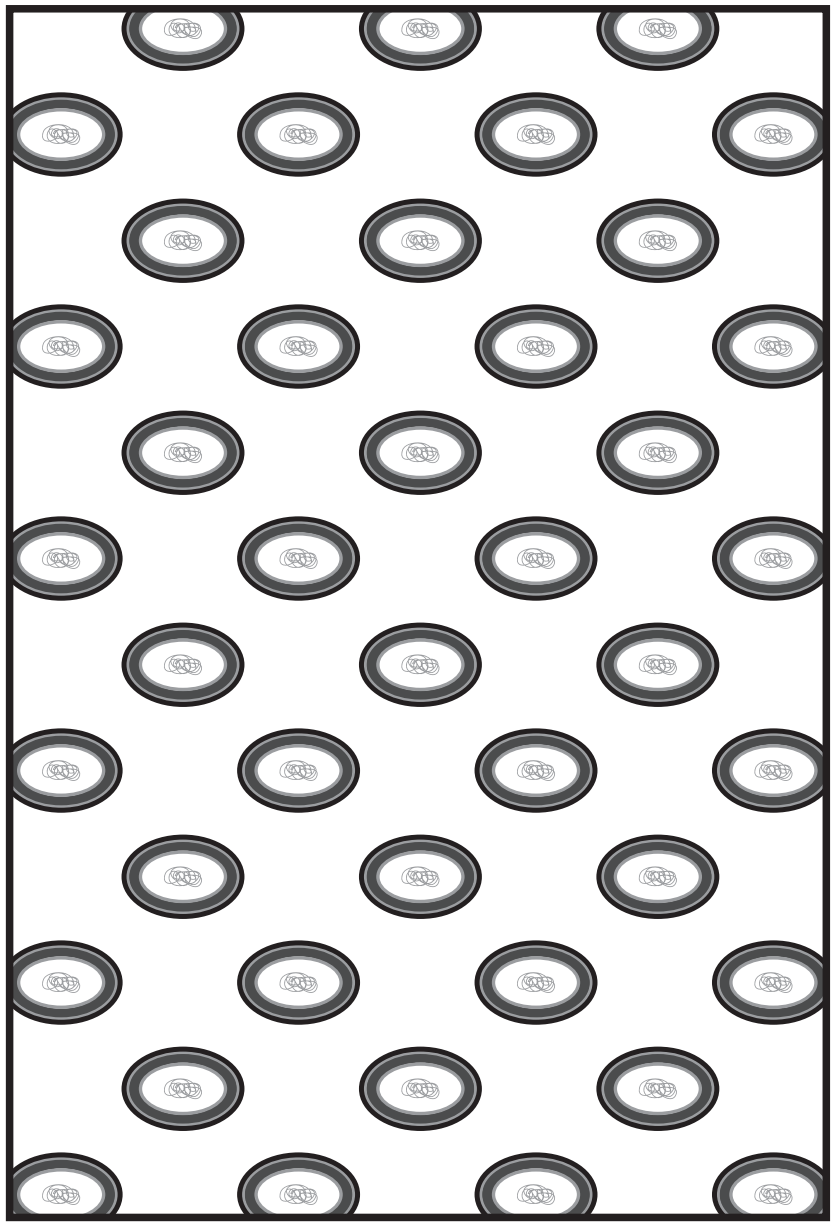

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Summary and general discussion

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Members of the *Bacillus* genus can respond to unfavorable environmental conditions such as low nutrient levels and high cellular concentrations by an array of survival strategies, including motility, genetic competence, biofilm formation, cannibalism or spore formation (1–3). These cellular differentiation programs are coupled to each other, which can be manifested by their mutual exclusivity or occurrence in a sequential order. Sporulation, which is highly time- and energy-consuming (4), constitute the last resort survival strategy to starvation, leading to the formation of metabolically dormant (endo)spores (Figure 1), that represent one of the sturdiest known forms of life (5–7).

Because of their unique resistance and abundant occurrence in soil, spores easily contaminate natural materials and industrial facilities, including the food production chain, from which they are difficult to eradicate (Figure 1) (8–10). This causes problems for the food industry, as food processing treatments used are often insufficient for complete inactivation of contaminating spores. Despite dormancy, spores sense their environment and in response to specific triggers can restore vegetative growth via the processes of germination (11, 12) and outgrowth (13–15). If the revival of vegetative growth occurs in food, it can lead to its spoilage (Figure 1) and, in the case of pathogenic species such as *B. cereus*, spreading of foodborne diseases (16, 17).

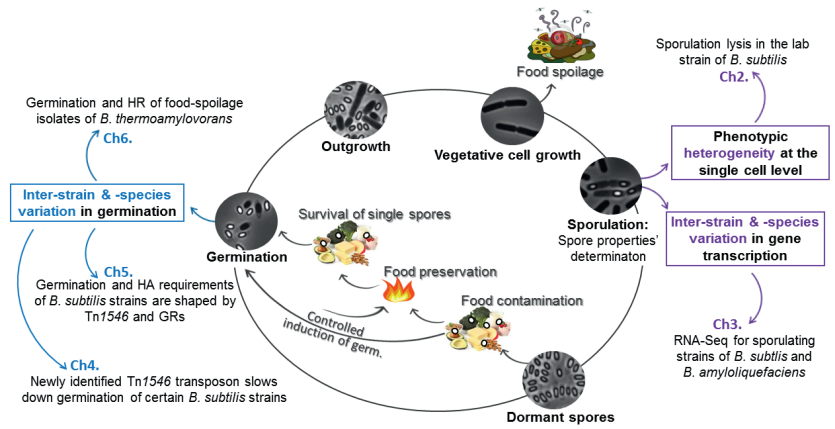

Figure 1. Overview of research themes studied in Chapters 2 to 6 (Ch2-Ch6) of this thesis in the context of the *Bacillus* sporulation life cycle and its importance to the food industry. Abbreviations: Ch – chapter; germ. – germination; HA – heat-activation; HR – heat resistance.

Diversity in sporulation and spore properties

This thesis focuses on diversity in sporulation (**Chapter 2 and 3**) and spore properties, in particular spore germination characteristics (**Chapter 4-6**). This diversity comprises both heterogeneity at the single cell level in monoclonal populations (**Chapter 2**) and variation between different species and strains (**Chapter 3-6**). Both single-cell heterogeneity as well as inter-strain and -species variation complicates prediction of the spore behavior and hinders standardization of the inactivation procedures applied by the food industry. Thus, to assess the extent and origin of the diversity in spore properties, research described in this thesis includes both a common laboratory organism, *B. subtilis* 168 (**Chapter 2-6**), environmental isolates of *B. subtilis* (**Chapter 4 and 5**) and industrially-relevant food-spoilage isolates *of B. subtilis* (**Chapter 3-5**), *B. amyloliquefaciens* (**Chapter 3**) and *B. thermoamylovorans* (**Chapter 6**).

Most of knowledge on sporulation and spore characteristics originates from studies performed on model laboratory strains (in particular the non-pathogenic soil-derived *B. subtilis* 168), which are relatively easy to work with due to the existance of well developed molecular and genetic manipulation tools. Yet, such strains have commonly lost some of their original traits during the domestication process and cultivation under laboratory conditions (18–20). Moreover, they do not cover the genetic and phenotypic diversity encountered in nature and have distinct properties from environmental isolates, especially the ones derived from extreme environments or selected by industrial processes (**Chapter 3-6**). For this reason, the direct translation of knowledge from laboratory strains to natural or industrially relevant isolates is often impossible, even within the same species. For instance, as indicated by expression data for eight strains from the *B. subtilis* group, the food-spoilage isolates may have a different bias towards the individual lifestyles and survival strategies than the laboratory model strain 168 (**Chapter 3**), making an investigation of their adaptive responses other than sporulation an interesting subject for future investigation. The observed variation likely originates from the adaptation of individual strains and species to specific environmental conditions. Consistently, the largest differences were observed in transcription and presence of genes involved in responses to the surrounding (e.g., genes involved in the initiation of spore formation and other differentiation programs or triggering germination) and protection against the adverse conditions (e.g., genes involved in coat and cortex assembly or various resistance mechanisms) (**Chapter 3**). For the same reason, spore properties that allow for a reaction to the environmental factors such as germination and heat resistance are highly variable among different species and strains (**Chapter 4-6**) (21). Moreover, heterogeneity among individual genetically identical spores, which might be partly caused by stochastic fluctuations in gene expression, may also play an adaptive role as it increases chances of survival of a part of a population in a changeable and unpredictable environment (**Chapter 2**) (22).

Variation in spore germination

Spore germination phenotypes, on which a large part of this thesis is focused, show a huge inter-species and inter-strain variation (**Chapter 4-6**), which includes: i) the type of nutrient and non-nutrient germinants that spores are responsive to (**Chapter 4-6**) (23–26); ii) the required germinant concentration (**Chapter 6**) (27, 28); iii) the spore requirements for the prior heat-activation treatment that increases their responsiveness to nutrient germinants (**Chapter 5**) (29); iv) the germination efficiency reflected by a number of spores in a population that germinate upon exposure to a specific trigger (**Chapter 4-6**) (27, 28, 30–32); v) germination rate and kinetics (**Chapter 4-6**) (23, 27, 28, 30); and vi) the influence of general environmental/experimental conditions such as pH, temperature or salt concentration (**Chapter 6 and unpublished data**) (27). In some cases such as in the examples described below, the molecular bases of the phenotypic differences observed in germination of spores of different species and strains are (partly) known, while in others they still remain to be elucidated.

Presence and sequences of germinant receptor genes largely contribute to variation in spore germination responses to nutrients

The presence and sequences of germinant receptor complexes (GRs) constitute important factors that vastly contribute to the differences in spore responsiveness to various nutrient germinants. GRs sense specific nutrient compounds and transfer a signal of unknown nature to the downstream germination effectors (33), in particular the SpoVA channel (34–36). GRs usually consist of three or four subunits (A, B, C and D), often encoded within one *ger* operon (37–39), with B subunits likely being responsible for germinant binding (26). Different species and strains contain various types and numbers of GRs, which vary in their substrate specificities and affinities (23, 37, 38) as well as in heat-activation requirements and/or thermal stabilities (**Chapter 5**) (29). The individual GRs can interact with each other within the spore IM (40), showing synergism (41) and positive and negative cooperativity (42–45). Additionally, incomplete *ger* operons can be found in

the spore-formers' genomes. In *B. megaterium*, monocistronic loci encode orphan B subunits, which are able to interact with A and C subunits of other GRs, extending a range of germination-triggering nutrients (25, 26). In turn, some *B. subtilis* strains contain an operon encoding solely A and C GR subunits, carried on a transposon-like element (**Chapter 4 and 5**). Although the functionality of these products is questionable due to the truncations of their sequences (**Chapter 4 and 5**), it cannot be excluded that they affect spore germination, for instance by interfering with other GR complexes, under specific, untested conditions.

Besides the presence of *ger* operons, specific amino-acid sequences of encoded subunits also affect GR specificity, affinity (**Chapter 5**) (26, 32, 46–48) and likely heat-activation requirements and/or thermal stabilities (**Chapter 5**). Various differences in the amino acid composition of the subunits of the GerA, GerB and GerK GRs of the 17 investigated *B. subtilis* strains correlated with their different responsiveness to L-alanine, the AGFK (L-asparagine, glucose, fructose and K^+) co-germinant mixture (**Chapter 5**) and the nutrient-rich LB medium (**Chapter 4**). This is coherent with previous studies that have shown that even a single point mutation can lead to changes in spore germination efficiencies, kinetics, sensitivity to germinants and requirements for positive cooperativity with other GRs in order to initiate germination (33, 42, 46–49). In another study on multiple *B. subtilis* strains (28), the similar relatively high inter-strain variation in GerB protein sequences has been proposed to account for the diversity in spore responsiveness to AGFK (28). Consistently, our study revealed a lower conservation of the protein sequences for GerB and GerK than for GerA and less preserved ability for efficient germination in AGFK than in L-alanine (**Chapter 5**).

The GR protein sequences of the 17 *B. subtilis* strains also seem to affect the sensitivity of the specific spore germination pathways to heat (**Chapter 5**). Differences in the GR amino-acid composition likely change the effect of different heat-activation conditions on spore germination with the specific nutrient triggers. Moreover, they may simultaneously lead to a lower activity of the thermostable variants of GRs when compared to the thermosensitive ones due to an increase in protein rigidity, as it has been observed for other types of proteins (50, 51). These potentially thermostabilizing substitutions occur more commonly in the *B. subtilis* strains that produce more heat resistant spores, suggesting co-evolution of these two features. Possibly, the increased level of spore heat resistance has allowed for the accumulation of mutations that increase the thermal stability of GRs (**Chapter 5**). Introduction of these substitutions to the genetically accessible strains, i.e., *B. subtilis* 168 wild type (wt) that produces low-level heat resistant spores and to its derivative B4417 (or 168HR) that produces high-level heat resistant spores (**Chapter 4**) (21) would allow for assessment of their potential effects on activities and thermal stabilities of the individual GR-mediated germination pathways.

Certain strains contain the *ger* operons that are weakly transcribed (**Chapter 3 and 5**) (33) and lack any detectable activity in the tested conditions (**Chapter 5 and 6**) (28, 33). Potentially, due to the accumulated mutations, such operons have (partly) lost functionality and hence, play at best a minor, auxiliary, role in spore germination. For instance, the tricistronic *ynd* operon is poorly expressed and is likely inactive in the laboratory strain *B. subtilis* 168 (33). However, this might not apply to certain foodborne strains, in which *ynd* is transcribed more strongly during sporulation and has slightly different sequences (**Chapter 5**). Thus, it would be interesting to verify if the putative Ynd GR of these strains has an ability to trigger germination, either independently or cooperatively with other GRs, as described previously for Ynd in the closely related *B. licheniformis* (52). Further investigation is also required to (dis)proof functionality of GerA in *B. subtilis* B4067 and B4145, which produced spores irresponsive to L-alanine in our experimental conditions (**Chapter 5**), and of the two putative GRs in *B. thermoamylovorans* spores, which showed no responses to any of the tested nutrient mixes (**Chapter 6**). Similarly, it would be worth to investigate whether AGFK-induced germination observed for part (up to ~38%) of B4067 and B4145 spores is mediated by products of the *gerB* operon, which contains mutations that influence length and structure of the encoded proteins (**Chapter 5**). Alternatively, the GerK receptor of these strains could trigger germination independently or in cooperation with GerA instead of Ger*B*.

Genes for CLEs likely contribute to variation in spore germination with the non-nutrient germinant, Ca-DPA

Besides GRs, also differences in presence and sequences of genes encoding other germination-related proteins can lead to diversity in spore germination phenotypes. For instance, the presence of a second copy of the *cwlJ* (*cwlJ2*) and *gerQ* (*gerQ2*) genes in two *B. thermoamylovorans* strains investigated in this study is the most plausible reason for the observed two-fold stronger germination of their spores in the non-nutrient germinant, exogenous Ca-DPA, in comparison to spores of strains with only a single copy of *cwlJ* and *gerQ* (**Chapter 6**). The *cwlJ* gene encodes a cortex lytic enzyme (CLE) that is activated during the DPA passage from the spore core to the outside environment upon germination with nutrients (53). The GerQ protein is essential for proper localization of CwlJ in the outer spore coat (54).

While species such as *B. subtilis* and *B. cereus* are known to contain a single copy of both *cwlJ* and *gerQ*, the presence of two copies of *cwlJ*, encoding CwlJ1 and CwlJ2, has been previously reported for *B. anthracis* (55, 56). In *B. anthracis*, the *cwlJ2* gene is relatively poorly transcribed, lacks the accompanying *gerQ2* gene and its effect on germination is predominantly visible in the ΔcwlJ1 ΔsleB mutant with a deletion of other CLEs (55, 56). It is possible that the simultaneous presence of *cwlJ2* and *gerQ2* is important for a more pronounced role of CwlJ2 in Ca-DPA-induced spore germination of *B. thermoamylovorans*. Development of tools for genetic manipulation of *B. thermoamylovorans* would allow to directly test this possibility, while transcriptomic data for all four genes would provide an additional indirect support. Moreover, investigation of differences in nutrient-induced spore germination for the *B. thermoamylovorans* strains containing one or two copies of *cwlJ* and *gerQ* would provide clues on the role of these genes in more physiologically-relevant conditions. Unfortunately, such analysis is currently hindered by a lack of known efficient nutrient germination triggers for this species (**Chapter 6**).

The newly identified Tn*1546* transposon and *spoVA*^{2mob} operon constitute a common ground of variation in spore germination and heat resistance properties

The *spoVA*^{2mob} operon identified parallelly in two studies (**Chapter 4**) (21) via a gene-trait matching approach constitutes another factor that strongly contributes to the variation in several aspects of spore germination (**Chapter 4-6**) and heat resistance (21). The name of this operon used here refers to its partial homology to the *spoVA* operon that is conserved among bacilli spore-formers ("*spoVA*^{2}") and to its location on the mobile genetic element ("mob") (see below).

The *spoVA*^{2mob} operon was initially found in ten food-spoilage isolates of *B. subtilis* as part of a Tn*1546*-like transposon element, which is absent in the common laboratory strain 168 (**Chapter 4**) (21). *spoVA*^{2mob} is present in up to three copies in genomes of some *B. subtilis* strains: two copies on the two Tn*1546* elements (integrated within the *yitF* gene and additionally between the *yxjA* and *yxjB* genes) and one Tn*1546*-independent copy in an unknown genomic context (21).

The presence of the Tn*1546*-like transposon and the *spoVA*^{2mob} operon decreases the rates and efficiencies of nutrient-induced spore germination, measured as a decrease in optical density of spore suspensions or as a DPA release from spores upon germination (**Chapter 4 and 5**). Tn*1546* and *spoVA*^{2mob} also decrease spore responses to a non-nutrient germinant, the surfactant dodecylamine, while they have no visible effect on germination with exogenous Ca-DPA (**Chapter 4**). Moreover, as indicated by correlation, the *spoVA*^{2mob} products likely increase spore heat-activation requirements for nutrient-induced germination (**Chapter 5**). Finally, the presence of this operon in *B. subtilis* strains leads to a strong increase in the heat resistance level of produced spores, when compared to spores of strains that do not contain *spoVA*^{2mob} (21).

The effects of *spoVA*^{2mob} on nutrient-induced germination, spore heat-activation requirements (**Chapter 5**) and heat resistance properties (21) appear to increase with a higher copy number of *spoVA*^{2mob} (and Tn*1546*).

Products of *spoVA*^{2mob} seemingly affect Ca-DPA transport

The *spoVA*^{2mob} operon carried on Tn*1546* in certain *B. subtilis* strains consists of seven genes (Figure 2A) that subsequently encode: i) a protein of unknown function containing a DUF1657 domain; ii) a putative lipoprotein with a YhcN/YlaJ domain; iii) SpoVAC^{2mob}; iv) SpoVAD^{2mob}; v) SpoVAEb^{2mob}; vi) a second protein of unknown function with a DUF1657 domain; and vii) a protein of unknown function with DUF421 and DUF1657 domains (**Chapter 4**) (21). Three of these proteins, SpoVAC^{2mob}, SpoVAD^{2mob} and SpoVAEb^{2mob}, are homologous to SpoVAC, SpoVAD and SpoVAEb, respectively, encoded on the core genome heptacistronic *spoVA* (*spoVAA-spoVAB-spoVAC-spoVAD-spoVAEb-spoVAEa-spoVAF*) operon (Figure 2B), long known from the research on the laboratory model strain of *B. subtilis* (34, 57, 58). In contrast to *spoVA*^{2mob}, the presence of the *spoVA* operon is conserved in spore-forming bacilli (11, 21, 59), which is in line with its essential role in spore formation (36). The products of the "regular" *spoVA* operon are required for the uptake of dipicolinic acid (DPA) to the forespore during spore formation, which is important for a dehydrated state of the spore core and spore resistance properties (36). Additionally, the operon plays a role in the release of DPA via the spore inner membrane (IM) upon germination with nutrients and dodecylamine (34, 35, 60–62).

The function of the individual products of the conserved *spoVA* operon is not fully understood. All of them are predicted or documented spore IM proteins, with SpoVAA, SpoVAC and SpoVAF having a proven localization in the IM and SpoVAD and SpoVAEb reportedly present on the IM outer surface (36, 62–65). SpoVAC likely acts as a main component of a mechanosensitive channel in the spore IM; such channel may react to changes in the membrane tension and osmotic pressure (35). SpoVAD has an ability to

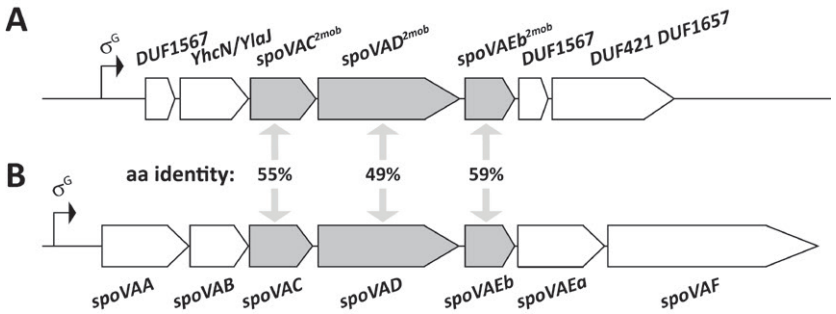

Figure 2. Comparison of the *spoVA*^{2mob} operon from the Tn*1546* transposon of certain *B. subtilis* strains (A) with the "regular" *spoVA* operon, conserved among spore-forming bacilli (B). Percentages of amino acid sequence identity (aa identity) between the three homologous proteins encoded in the two operons are indicated. The figure is adapted from (21).

directly bind DPA (36). Mutations in any of its first five *spoVA* cistrons (Figure 2B) abolish DPA uptake and prevent completion of spore formation (36, 62). In contrast, *B. subtilis* strains that lack SpoVAF or SpoVAEa and SpoVAF, encoded by the two final genes (Figure 2B), sporulate normally and produce spores with regular DPA levels (62). Still, their spores respond more slowly, exhibit longer T_{lag} between the addition of nutrients and the rapid DPA release and likely need more time for commitment upon germination with GR-dependent germinants, such as nutrients and high hydrostatic pressure (HHP) of ~150 MPa, when compared to wild-type spores (62). They also exhibit slightly slower germination with HHP of ~550 MPa, while their responses to dodecylamine are unaffected. Both HHP of 550 MPa and dodecylamine trigger germination predominantly via the SpoVA channels (62).

SpoVAC^{2mob}, SpoVAD^{2mob} and SpoVAEb^{2mob} encoded in the *spoVA*^{2mob} operon present on Tn*1546* show 55%, 49%, and 59% of amino-acid sequence identity, respectively, to SpoVAC, SpoVAD and SpoVAEb encoded in the core genome *spoVA* operon (Figure 2) (**Chapter 4 and 5**) (21). This similarity suggests a potential auxiliary role of the *spoVA*^{2mob} products in DPA transport across the spore IM. Consistently with this hypothesis, spores of *B. subtilis* 168 with the Tn*1546* element integrated within the native *yitF* locus (B4417 strain) or with *spoVA*^{2mob} inserted in the ectopic *amyE* locus (168 *amyE*::*spoVA*^{2mob} strain) contain a 1.5-fold higher DPA concentration than wild-type 168 spores (21), indicating increased DPA transport to the spore during sporulation. The observed increase in heat resistance in spores of strains containing *spoVA*^{2mob} may be (at least in part) directly caused by this higher core DPA content. Indeed, accumulation of DPA in the spore has been associated with a low core water content required for resistance of spores to wet heat (7, 66). Moreover, as spores with low DPA content are more susceptible to spontaneous germination in the absence of germinants (67, 68), it is possible that these higher DPA concentrations also directly lead to an increase in spore dormancy and stability.

In contrast to DPA uptake during sporulation, DPA efflux during germination with nutrients or dodecylamine was slower and/or less efficient for B4417 spores (1.5-2.1-fold less $DPA_{release}/DPA_{total}$ upon AGFK-induced germination) when compared to B4417Δ*spoVA*^{2mob}, B4417ΔTn*1546* and 168 wt spores (**Chapter 5** and unpublished data). Weaker responses to nutrient germinants were also observed for spores of strains that contain Tn*1546* and *spoVA*^{2mob} when germination was measured as a loss of optical density (OD_{600}), which corresponds to rehydration of spores (**Chapter 4 and 5**). These trends were visible both for intact spores and for spores that were subjected to coat removal prior to exposure to nutrients (**Chapter 4**). Opposite to nutrient- and dodecylamine-induced germination, the presence of Tn*1546* and *spoVA*^{2mob} did not visibly affect spore germination in response to exogenous Ca-DPA, which directly initiates cortex hydrolysis (69) (**Chapter 4**). Altogether, these data suggest that despite causing a potential increase in DPA uptake to the spore during sporulation (21), Tn*1546* and *spoVA*^{2mob} have a negative impact on the DPA release and possibly other early aspects of germination, such as sensing nutrients by GRs or transfer of a signal from GRs to the SpoVA channel. In contrast, these genetic elements do not seem to significantly affect germination at the stages of the passage of nutrients through the spore coat (70) and during cortex hydrolysis (53).

The seemingly opposite effect of *spoVA*^{2mob} on DPA transport across the (fore)spore IM during sporulation and germination suggests that these two processes have somewhat different molecular mechanisms and can be influenced differently by the same factors. Such differences may be related to the opposite direction of the DPA movement, distinct properties of the IM in the still immature forespores and in the mature dormant spores and/or a requirement for GRs in order to initiate DPA release during germination. Thus, the *spoVA*^{2mob} products seem to support the DPA transport across the IM during sporulation, but not during germination. One possibility is that the *spoVA*^{2mob}-encoded proteins build an alternative type of mechanosensitive channels, which only transport DPA inwards during sporulation. During germination, these unidirectional channels would compete with the "regular" SpoVA channels for DPA binding, ultimately hindering DPA release. In contrast to *spoVA*, the *spoVA*^{2mob} operon does not encode homologs of SpoVAEa and SpoVAF, which are reportedly important for GR-dependent spore germination (62). SpoVAF shows a significant sequence homology to the GR A subunits (57, 71), and thus might be involved in the interactions between the GRs and the IM channels. Therefore, the potential SpoVA^{2mob}

channels, lacking the SpoVAF counterpart, might be irresponsive to the germination signals sent by the GRs. Alternatively, the products of *spoVA*^{2mob} could interact with the "regular" SpoVA proteins, forming hybrid channels that do not support DPA transport during germination, for instance due to an imbalance in the number of SpoVAC, SpoVAD and SpoVAEb subunits that are encoded in both operons and the number of the remaining subunits that are encoded solely on *spoVA*. Finally, as five of the *spoVA*^{2mob} products (encoded by 2nd to 5th and 7th gene) are predictively associated with the spore IM, they could potentially affect the IM properties, which have previously been shown important for both the spore germination capacities and the levels of spore resistance to wet heat (72). As heat-activation of spores has been hypothesized to enhance germination by acting either on the spore IM, the GRs or both (**Chapter 5**) (29), an increase in spore heat-activation requirements caused by *spoVA*^{2mob} (**Chapter 5**) supports both the possibility that *spoVA*^{2mob} alters the IM properties as well as the communication between the GRs and the DPA channels in the IM.

Next to the three SpoVA homologs, (some of) the four proteins of unknown functions encoded on *spoVA*^{2mob} could play a role in decreased spore germination. Unfortunately, as they do not share sequence homology with any well-studied proteins, prediction of their biological functions is difficult. The products of the 1st and 6th gene that contain the DUF1657 domain do not have any predicted membrane-bounding sequences and, hence, are likely cytosolic. The product of the 2nd gene is a predicted lipoprotein attached to the IM by an added lipid anchor, similarly to the known germination-related lipoproteins GerD and C subunits of GRs (64, 73). The product of the last, 7th, gene contains both the DUF1657 domain as well as the N-terminal DUF421 domain. According to a topology prediction by TOPCONS (74), this protein penetrates the (spore inner) membrane via three transmembrane (TM) helices localized within the initial ~80 amino-acids of DUF421, while the remaining part of the protein resides inside the IM. This predicted IM-associated localization of the putative lipoprotein and of the DUF421-DUF1657 protein encoded by the 2nd and 7th gene, respectively, could potentially enable these proteins a direct interaction with the IM-localized SpoVA channel, GerD and GR complexes. The forespore-specific synthesis has recently been confirmed for another DUF421-containing protein, YetF (75). As indicated by Conserved Domain Architecture Retrieval Tool (CDART, https://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps) (76), the predicted membrane-spanning DUF421 domain can occur alone or be accompanied by other domains, such as PIN or domains involved in ligand/substrate-binding (such as HTH, NADB, zinc ribbon or periplasmic binding domains) in proteins that might participate in signaling, stress response, ligand translocation across the membrane or (transcriptional) regulation. In turn, the DUF1657 domain is found within proteins that contain predicted mechanosensitive ion channel domains, for instance in a putative potassium transporter. Thus, conceivably, the DUF421-DUF1657 integral membrane protein could function as a sensing or ligand-binding membrane component of a mechanosensitive channel with a role in DPA transport. Moreover, the DUF1567 domains of the cytosolic products of the 1st and 6th gene of *spoVA*^{2mob} could allow for interactions between these proteins and DUF421-DUF1657.

Indeed, deletion of the last, 7th, gene that encodes a protein with the DUF421 and DUF1567 domains from B4417 (B4417Δ*2DUF*) strain improved spore germination, however to a lower extent than deletion of the whole *spoVA*^{2mob} operon (**Chapter 4**). Moreover, deletion of this gene has been shown to abolish high-level heat resistance of spores, although its sole insertion without the first six genes of *spoVA*^{2mob} has not rendered high-level heat resistant spores (21). Altogether, these data suggest that the last gene of the *spoVA*^{2mob} operon may play a role in decreased spore germination and especially in elevated spore heat resistance. However, other factors encoded on *spoVA*^{2mob}, and possibly also on other operons of Tn*1546*, are required for the maximal expression of these phenotypes.

Analysis of the effects of deletions of single genes of the *spoVA*^{2mob} operon on spore germination in response to different nutrients, dodecylamine and HHP, which can trigger germination response either via GRs or the SpoVA channels (62), and on spore heat resistance could reveal which of these genes are predominantly involved in the alternation of these spore properties. Moreover, sporulation assays should be performed on B4417 and of *B. subtilis* 168 *amyE*::*spoVA*^{2mob} strains with a deletion of the core genome *spoVA* operon. Such experiments would establish whether the *spoVA*^{2mob} operon on its own is sufficient for completion of sporulation and accumulation of the adequate spore core levels of DPA. Sufficiency of *spoVA*^{2mob} for the formation of viable, dormant and resistant spores would indicate that its products are able to form self-sufficient channels in the spore IM, which are independent of the "regular" SpoVA proteins. Moreover, germination assays performed on spores produced by the strains that contain *spoVA*^{2mob}, but lack *spoVA*, would uncover if the *spoVA*^{2mob} operon alone is capable of triggering spore germination responses. It is an especially interesting question, taking into account that the *spoVA*^{2mob} operon does not encode homologs for four of the *spoVA* genes, including *spoVAEa* and *spoVAF* with proven roles in germination (62). Finally, comparison of the effects of the *spoVA*^{2mob} operon in the ΔspoVA background on DNA uptake during sporulation and release during germination could provide data on molecular differences distinguishing these two processes.

Bacillaceae contain up to three types of the *spoVA*-like operons

A previous phylogenetic study (21) revealed the existence of up to three types of *spoVA*-like operons, which belong to two different phylogenetic groups, among 103 spore-forming Bacillaceae. The first phylogenetically separate type constitutes the conserved "regular" *spoVA* operon with seven genes ranging from *spoVAA* to *spoVAF*, described above and studied in detail in *B. subtilis* (Figure 2B) (34, 36, 61, 62). The second and third type comprise *spoVA*^{2} and the described-above *spoVA*^{2mob} that belong to the same phylogenetic group and both contain the same four genes of unknown function and three orthologs of *spoVAC*, *spoVAD* and *spoVAEb* (Figure 2A) (**Chapter 4**) (21). In contrast to *spoVA*^{2mob}, which is present specifically on mobile genetic elements, such as the Tn*1546* transposon, the *spoVA*^{2} occurs as a part of the core genome (21).

Conserved "regular" *spoVA* (11, 59) is present in the all analyzed Bacillaceae strains (21). Although certain species, i.e., *B. thermoamylovorans* (**Chapter 6**), *Bacillus sporothermodurans* and *Geobacillus debilis*, have lost 3 to 6 of the *spoVA* genes (always with an exception of germination-involved *spoVAF*) (21); the effects of such loss might be complemented by the presence of the *spoVA*^{2} and/or *spoVA*^{2mob} operons in their genomes. Consistently, *spoVA*^{2} has been found in all analyzed species (*B. thermoamylovorans*, *B. sporothermodurans*, *Geobacillus spp*., *Anoxibacillus flavithermus* and *B. cereus*) except for the *B. subtilis* group (*B. subtilis*, *B. licheniformis* and *B. amyloliquefaciens*) (21).

The *spoVA*^{2mob} operon has been proposed to originate from the duplication of chromosomally-located *spoVA*^{2} onto the pXOI-like plasmid of some *B. cereus* strains, which also contains the Tn*1546* transposon (21). Subsequently, it has been hypothesized to be able to spread by horizontal gene transfer to other species, including part of the analyzed *B. subtilis*, *B. amyloliquefaciens* (containing 1-3 copies of *spoVA*^{2mob}) and *B. licheniformis* (1 copy) strains, *B. thermoamylovorans* (1 complete and 1 incomplete copy) and *B. sporothermodurans* (1 copy) (21).

All analyzed strains contain at least one copy of *spoVAC*-, *spoVAD*-, *spoVAEb*- and *spoVAF*-type genes, encoded on *spoVA*, *spoVA*^{2} and/or *spoVA*^{2mob} (21). Moreover, a vast majority of them (all except *G. debilis*) contain also genes coding for SpoVAA and SpoVAB within the *spoVA* operon. Thus, these genes seem to be crucial for DPA uptake and spore formation and in the case of *spoVAF* and possibly *spoVAA* and *spoVAB*—(also) for DPA release during germination (62). Analysis of sporulation and germination of *G. debilis* could provide clues about potential adjustments in DPA transport that compensate for the absence of *spoVAA* and *spoVAB*, which in *B. subtilis* are required for sporulation (36, 62).

Effects of *spoVA*^{2mob} on spore germination and heat-resistance observed in *B. subtilis* (**Chapter 3 and 4**) (21) are likely universal across different species. Thus, the presence of the two *spoVA*^{2mob} operons, next to an incomplete *spoVA* (*spoVAA*-VAB-VAF) and *spoVA*^{2} (21), likely accounts for the exceptionally weak germination of *B. thermoamylovorans* spores in response to nutrient triggers (up to ~2-15% germinated spores) despite the presence of two complete GR operons (**Chapter 6**). Consistently, these spores exhibited also high-level heat resistance (**Chapter 6**). Similarly, *B. amyloliquefaciens* B425 that contains *spoVA*^{2mob} and produces high-level heat resistant spores (21, 77) exhibits relatively weak spore germination with nutrients (unpublished data). Finally, preliminary data suggest that insertion of *spoVA*^{2mob} cloned from *B. licheniformis* and *B. amyloliquefaciens* strains into the *B. subtilis* 168 *amyE* locus leads to a decrease in spore germination (unpublished data), similarly as in the case of insertion of *spoVA*^{2mob} cloned from the *B. subtilis* B4067 food isolate (**Chapter 4**). Future work should study the effect of *spoVA*^{2mob} on spore properties in *B. cereus*, which constitutes a problematic pathogen causing foodborne diseases, is phylogenetically distant from the *B. subtilis* group (78) and predominantly produces low-level heat resistant spores (79). Moreover, an impact of the *spoVA*^{2} operon, which has a comparable composition and structure as *spoVA*^{2mob} but the chromosomal location (21), on spore germination and heat resistance should be investigated. *spoVA*^{2} has been found in a majority of tested species (21), including ones that form low-level heat resistant (79) and efficiently germinating spores (23, 27, 80). Hence, such analysis could reveal differences (and their origin) in roles of *spoVA*^{2} and *spoVA*^{2mob} in shaping spore properties. Potentially, the genetic context of the *spoVA*^{2mob} operon may play a role in the development of poor spore germination and high-level heat resistance phenotypes. Indeed, a possible impact of the genetic location is in line with the weaker phenotypic effects of the *spoVA*^{2mob} operon integrated alone in the ectopic *amyE* locus then of the *spoVA*^{2mob} present within the Tn*1546* element in the native *yitF* locus (**Chapter 4**) (21).

Spore heat resistance, germination and heat-activation requirements are inter-connected

The results presented in this thesis (**Chapter 4-6**) combined with studies on spore heat resistance (21, 77) indicate that the *spoVA*^{2mob} operon constitutes a clear genetic link between high-level spore heat resistance, high spore heat-activation requirements and lower rates and efficiencies of spore germination. All these spore characteristics could improve survival of a spore-former population in certain, especially extreme and changeable,

environments. It still remains to be elucidated if exactly the same (combinations of) genes encoded on *spoVA*^{2mob} and the same molecular mechanisms equally contribute to the alternations in all of these spore characteristics. Certain mechanistic differences appear plausible considering a potential requirement of the last gene of *spoVA*^{2mob} for the high-level heat resistance (21) and a rather weak effect of its deletion on the spore germination rates (**Chapter 4**).

An anti-correlation between the spore heat resistance levels and germination rates and efficiencies has been also observed for spores of strains that lack the *spoVA*^{2mob} operon (81–83). For instance, conditions during sporulation that lead to elevated levels of spore heat resistance, possibly by altering the IM properties (82), simultaneously negatively influence spore germination (81, 82). Moreover, superdormancy (SD) of spores of the laboratory strains of *B. subtilis*, *B. cereus* and *B. megaterium* has been shown to coincide with their higher heat resistance levels and heat-activation requirements (83). The superdormant (SD) spores reportedly have lower core water content (83), which is a known factor playing a role in spore resistance to wet heat (7), the different spore core environment of DPA (83) and lower protein levels of the specific GRs (84) than the dormant spores. It would be interesting to elucidate whether the mechanisms of increased heat resistance and decreased germination responses in (superdormant) spores that do not contain *spoVA*^{2mob} are analogous to the action of the *spoVA*^{2mob} operon in Tn*1546*-harboring strains tested in our study (**Chapter 4 and 5**).

Candidate factors that possibly contribute to variation in spore germination

Other, still unidentified factors are likely also involved in the determination of spore germination behavior and variation therein. For instance, a deletion of the entire Tn*1546* transposon from B4417 spores increased spore germination rates under certain conditions to a somewhat stronger level than a deletion of the single *spoVA*^{2mob} operon (**Chapter 4**), suggesting that some of the remaining four operons of Tn*1546* might contribute to the slowdown of spore germination. All of the genes carried on Tn*1546*, except for the fourth operon with the split, rather inactive *yetF* gene were expressed during spore formation (**Chapter 3 and 4**), thereby potentially affecting spore properties. However, individual deletions of these operons from the B4417 strain and their insertion in the *amyE* locus in *B. subtilis* 168 did not have a significant effect on spore germination, suggesting that their effects, if any, are only auxiliary to the role of the *spoVA*^{2mob} operon. Investigation of double deletion strains may reveal a potentially supporting role of operons 1, 2 or 5 of Tn*1546*.

Notably, GerA-mediated germination of B4417ΔTn spores was seemingly weaker than germination of *B. subtilis* 168 spores, indicating an effect of the genomic differences in the 100 kb chromosomal region between the *yitB* and *metC* genes (**Chapter 4**). Next to the presence of Tn*1546*, multiple single nucleotide polymorphisms (SNPs) distinguish the *yitB-metC* genomic region of *B. subtilis* 168 (85) and B4417; these SNPs are a result of the transduction method used for DNA transfer from the food isolate B4067 to 168 during creation of B4417 (**Chapter 4**) (21). Some of the SNPs led to truncations of genes encoded in this chromosomal region due to the frameshifts and nonsense mutations. Genes whose functionality might be abolished or compromised due to SNPs in the B4417 strain include *yitB*, *yitG*, *yitL* and *prpE*. Moreover, in contrast to *B. subtilis* 168, the *appA* gene in B4417 is intact and likely active. Out of these genes *yitB*, *yitG*, *prpE* and *appA* are expressed during spore formation (86). While the *yitG* deletion did not affect spore germination behavior (**Chapter 4**) and the active *appA* gene, encoding a subunit of the oligopeptide ABC transporter, rather affects sporulation initiation than spore germination (87, 88), a putative role of σ^K-controlled *yitB* (89, 90) and of *prpE* in spore germination is worth further investigation. Especially, *prpE* that encodes a protein tyrosine phosphatase, has been previously suggested to weaken GR-mediated germination (especially via GerA) by decreasing *gerA* and *gerK* transcriptional levels (91). An effect of *prpE* on expression levels of the *ger* operons has been later contradicted (92). Yet, a described phenotype (91) is in line with weaker GerA-mediated germination of B4417ΔTn spores when compared to 168 wt (**Chapter 4**) and with the lack of responses to L-alanine that was observed for spores of B4067 isolate despite the regular *gerA* expression levels in this strain (**Chapter 5**).

In addition to Tn*1546*, the presence of another group of genes in four operons (encoding a putative AzlC-like permease, a putative transcriptional regulator, a hypothetical protein, a predicted O-acetylhomoserine sulfhydrylase and a predicted fatty acid desaturase) correlated with slow spore germination (**Chapter 4**). Although insertions of single operons or pairs of these operons into the *B. subtilis* 168 *amyE* locus did not alter spore germination behavior (**Chapter 4**), it cannot be excluded that the presence of all these genes simultaneously or in the native genomic context might be required for the development of the phenotype. Moreover, other genes whose presence or absence correlated with a specific germination phenotype (slow/fast) (**Chapter 4** and unpublished data), indicated by the Phenolink software (93), such as the *yfkRST* putative GR operon (33) or the *pks* genes involved in polyketide synthesis (94) may have an impact on spore germination behavior.

Differences in expression of germination genes during sporulation may lead to single-spore heterogeneity in spore properties

Germination behavior differs not only between the spores produced by distinct bacterial strains and species but also between single spores in genetically identical populations. Especially the time (T_{lag}) between the addition of germinants and the fast Ca-DPA release is strongly variable between individual spores (95). The reasons for this variability are currently not well understood. It has been hypothesized that stochastic fluctuations in the expression of germination genes, especially the *ger* operons, which are dependent on the SpoVT and YlyA transcriptional regulators (96, 97), may be a contributing factor. However, this hypothesis has been contradicted by the finding that the heterogeneity in times required for germination of individual spores is unaffected by the changes in the *ger* expression levels (98). On the other hand, low levels of GRs have been associated with spore superdormancy to specific nutrients (84). Finally, the numbers of "activated" GRs and/or "SpoVA" channels, which are only partly coupled to the expression levels of the respective genes, may vary among the individual spores, reaching a threshold level required for germination only in some of them (61, 95).

In this work, we investigated heterogeneity in expression of various sporulation-specific genes, including multiple genes involved in germination such as *gerA*, *cwlJ*, *sleB*, *spoVA*, *gerP*, *gerE* (**Chapter 2**), *gerK* and *spoVT* (unpublished data). None of these genes showed a bistable expression pattern (low and high or off and on) in single sporulating cells. Yet, a certain variation in the continuously-distributed expression levels during sporulation was observed for almost all of these genes (**Chapter 2**). Lower expression levels appeared to be more common among cells that showed hallmarks of sporulation at a late time of microscopic observation and that lysed before completion of spore formation, presumably due to decreased cell viability (**Chapter 2**). It remains to be elucidated if the low expression levels in a subpopulation of cells that succeeded in spore formation correlate with the less efficient germination of the produced spores. This potential correlation could theoretically be caused by the lower level of a specific germination protein or be related to low fitness levels of spores that were produced by less viable cells. Unfortunately, in our time-lapse microscopy set-up (**Chapter 2**)(99), we were unable to assess germination behavior of the produced spores due to an inability to add germinants to the sealed microscopic slides. Possibly, use of another experimental set-up, for instance a microfluidic device (100, 101), would enable linking sporulation behavior and sporulation gene expression with the germination characteristics of produced spores.

Differences in expression of the *spoVA*^{2mob} operon (**Chapter 4 and 5**) (21) among individual sporulating cells of *spoVA*^{2mob}-harbouring strains could theoretically further increase single-spore heterogeneity in germination and heat resistant properties. Consistently, the preliminary time-lapse microscopic observation suggested the more broad distribution in times required for germination (transition from phase-bright to phase-dark spores) among the individual B4417 spores than for B4417ΔTn, B4417Δ*spoVA*^{2mob} and 168 wt (unpublished data), with more B4417 spores remaining dormant throughout the course of the experiment. Thus, the presence of *spoVA*^{2mob} could potentially increase the fraction of superdormant spores in a spore population. These hypotheses could be verified by analysis of *spoVA*^{2mob} expression on a transcriptional and post-transcriptional level and coupling it to the germination behavior of the individual spores.

Practical importance of this study for the food industry

The diversity in sporulation **(Chapter 3)** and spore properties **(Chapter 4-6)** described in this thesis underlines the importance of extending research from laboratory strains to natural and industrial isolates and of studying multiple strains in order to assess phenotypic and molecular variation in the investigated processes (**Chapter 3-6**). Especially, spore-formers isolated from environments in which they experience extreme conditions such as hot springs, deserts or foods subjected to processing and preservation treatments, which likely select for spores with specific properties, can provide valuable data on the adaptations of spore-formers to specific ecological niches. Coupling genomic and transcriptomic data to various phenotypic traits for multiple relatively closely related strains can elucidate the molecular bases of the observed inter-strain differences, similarly as in the case of the gene-trait matching approach used in this work (**Chapter 4**).

The observed diversity in spore properties poses problems for the standardization of food safety and risk assessment procedures. Plating methods that are often used for enumeration of spores in the food industry rely on the efficient spore germination, outgrowth and vegetative growth, needed for the colony formation. Therefore, the large inter-strain variation in the spore germination requirements underlines an importance of the use of strain-optimized germination and growth conditions when assessing spore counts by the plating methods. In particular, problematic, highly heat resistant spores tend to exhibit weak germination responses to common nutrient germinants (**Chapter 4-6**). Consequently, use of non-optimal germination conditions can lead to a severe underestimation of viable counts of such spores and to an

inaccurate assessment of their heat resistance properties (**Chapter 6**). These problems can be (partly) overcome by use of optimal strain-adjusted heat-activation treatments (**Chapter 5**) and/or alternative (combinations of) germinants such as Ca-DPA (**Chapter 6**) at the right concentrations. Importantly, the chosen conditions must also support the subsequent stages important for colony formation i.e., outgrowth and vegetative growth.

An identification of the *spoVA*^{2mob} operon (**Chapter 4**) (21) provides an explanation for part of the variation in spore germination and heat resistance that has been previously observed for other strains, including food spoilage isolates (**Chapter 6**) (20, 32, 77). For instance, the presence of *spoVA*^{2mob} likely accounts for the differences in spore germination rates observed among 46 strains of *B. licheniformis* that could not be formerly explained solely based on the GR amino-acid composition (32). Moreover, it allows for use of quantitative real-time PCR (qPCR) with the specific primers binding within *spoVA*^{2mob} for detection of spores with high-level heat resistance and slow germination characteristics, facilitating spore control and risk assessment in the industrial settings. Finally, a clear genetic link between spore heat resistance, germination and spore requirements for heat-activation needs to be considered when developing proper spore inactivation treatments as high temperatures used for spore inactivation may instead activate high-level heat resistant spores, facilitating their germination and subsequent food spoilage.

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